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Comparative vaccine efficacy of different isoforms of recombinant protective antigen against *Bacillus anthracis* spore challenge in rabbits

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Abstract

The next-generation human anthrax vaccine developed by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) is based upon purified *Bacillus anthracis* recombinant protective antigen (rPA) adsorbed to aluminum hydroxide adjuvant (Alhydrogel). In addition to being safe, and effective, it is important that such a vaccine be fully characterized. Four major protein isoforms detected in purified rPA by native PAGE during research and development were reduced to two primary isoforms in bulk material produced by an improved process performed under Good Manufacturing Practices (GMP). Analysis of both rPA preparations by a protein-isoaspartyl-methyltransferase assay (PIMT) revealed the presence of increasing amounts of iso-aspartic acid correlating with isoform content and suggesting deamidation as the source of rPA charge heterogeneity. Additional purification of GMP rPA by anion exchange chromatography separated and enriched the two principal isoforms. The in vitro and in vivo biological activities of each isoform were measured in comparison to the whole GMP preparation. There was no significant difference in the biological activity of each isoform compared to GMP rPA when analyzed in the presence of lethal factor using a macrophage lysis assay. Vaccination with the two individual isoforms revealed no differences in cytotoxicity neutralization antibody titers when compared to the GMP preparation although one isoform induced more anti-PA IgG antibody than the GMP material. Most importantly, each of the two isoforms as well as the whole GMP preparation protected 90–100% of rabbits challenged parenterally with 129 LD_{50} of *B. anthracis* Ames spores. The equivalent biological activity and vaccine efficacy of the two isoforms suggests that further processing to separate isoforms is unnecessary for continued testing of this next-generation anthrax vaccine.
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Keywords: Anthrax; *Bacillus anthracis*; Protective antigen; Vaccine; Isoforms; Rabbits

1. Introduction

The critical component of the new human anthrax vaccine developed at United States Army Medical Research Institute of Infectious Diseases (USAMRIID) is highly purified *Bacillus anthracis* recombinant protective antigen (rPA) that is combined with aluminum hydroxide (Alhydrogel). Several candidate vaccines based on rPA have been described [1–15] and have been shown to be highly protective against lethal aerosol or parenteral *B. anthracis* spore challenge.

rPA purified by a research and development (R&D) process is known to contain differentially charged forms (isoforms) of pure rPA protein with equivalent relative mass [16]. Micro-heterogeneity in PA structure was first described for protein purified from fermentor cultures of the Sterne strain grown in R-medium [17]. In an effort to optimize the yield of PA for vaccine production, the gene encoding PA (*pag*) was cloned into a *B. subtilis* strain [15] and, later, into the non-virulent, asporogenic Delta-Sterne-1 CR4 strain [18]. Although the Delta-Sterne-1 pPA102-CR4 strain produced rPA in higher yields, micro-heterogeneity was still observed [16]. Although the basis for the micro-heterogeneity in rPA is under investigation, the presence of a negatively charged

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14. ABSTRACT

The next-generation human anthrax vaccine developed by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) is based upon purified Bacillus anthracis recombinant protective antigen (rPA) adsorbed to aluminum hydroxide adjuvant (Alhydrogel). In addition to being safe, and effective, it is important that such a vaccine be fully characterized. Four major protein isoforms detected in purified rPA by native PAGE during research and development were reduced to two primary isoforms in bulk material produced by an improved process performed under Good Manufacturing Practices (GMP). Analysis of both rPA preparations by a protein-isoaspartyl-methyl-transferase assay (PIMT) revealed the presence of increasing amounts of iso-aspartic acid correlating with isoform content and suggesting deamidation as the source of rPA charge heterogeneity. Additional purification of GMP rPA by anion exchange chromatography separated and enriched the two principal isoforms. The in vitro and in vivo biological activities of each isoform were measured in comparison to the whole GMP preparation. There was no significant difference in the biological activity of each isoform compared to GMP rPA when analyzed in the presence of lethal factor using a macrophage lysis assay. Vaccination with the two individual isoforms revealed no differences in cytotoxicity neutralization antibody titers when compared to the GMP preparation although one isoform induced more anti-PA IgG antibody than the GMP material. Most importantly, each of the two isoforms as well as the whole GMP preparation protected 90-100% of rabbits challenged parenterally with 129 LD(50) of B. anthracis Ames spores. The equivalent biological activity and vaccine efficacy of the two isoforms suggests that further processing to separate isoforms is unnecessary for continued testing of this next-generation anthrax vaccine.

15. SUBJECT TERMS
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isoform is consistent with deamidation of amino acids containing a primary amine [19].

The non-enzymatic deamidation of asparagine (Asn) residues is one of the most common spontaneous modifications occurring during storage and manipulation of peptides and proteins [19]. A functional role for this phenomenon in vivo has been postulated [20]. Studies on model peptides show that the main reaction pathway involves the formation of a short-lived succinimide intermediate which hydrolyzes on either side of the imide nitrogen to generate iso-aspartic acid (isoAsp) and aspartic acid in approximately a 3:1 ratio [21]. The rate and extent of isoAsp formation varies widely and is greatly influenced by the amino acid sequence adjacent to the affected site, local polypeptide flexibility, and the degree and nature of solvation [22]. Observations that Asn deamidation and isoAsp content can alter the biological activity or immunogenicity of a protein [22,23] render the formation of isoforms during the development of biological drugs a serious consequence deserving further analysis.

In this study, we investigated whether rPA protein may contain deamidation as would be predicted by the presence of isoAsp, and describe changes in the protein purification process that yields a final product with fewer isoforms and lower isoAsp content. We also studied the biological activities and vaccine efficacy of purified isoforms as compared to the existing GMP final bulk product to better define the rPA substance in the new anthrax vaccine. We measured anti-rPA antibody responses by enzyme-linked immunosorbent assay (ELISA) and cytotoxicity neutralization (CN) assays [24,25], and protective efficacy by survival of rabbits challenged parenterally with virulent *B. anthracis* spores. Evaluating the protective efficacy of the two primary rPA isoforms compared to the GMP bulk product is critical for characterizing the candidate vaccine and will determine whether additional separation of the isoforms is necessary for continued product development.

2. Materials and methods

2.1. Proteins

Two preparations of rPA were used as vaccine antigens and for biochemical studies. Research and development grade rPA protein was prepared at USAMRIID as described previously [16], and a single lot (100506) of GMP rPA was prepared by the Biopharmaceutical Production Facility of SAIC at the NCI-FCRDC (Frederick, MD). GMP grade rPA was buffer exchanged into 25 mM ammonium acetate pH 6.8 (Buffer A) using Centriprep-30 concentrators (Millipore, Billerica, MA). The two major isoforms of GMP rPA were enriched by ion-exchange chromatography with two 6-ml Resource Q chromatography columns (Amersham Biosciences, Piscataway, NJ) connected in series. Chromatography was performed using an Akta Liquid Chromatography System (Amersham Biosciences). After the columns were equilibrated with 10 column volumes of Buffer A, the sample was applied at one column volume per minute, and the columns were washed with four column volumes of Buffer A. A 20-column volume linear gradient was applied to 70% of 300 mM ammonium acetate pH 6.8 (Buffer B). Fractions (3 ml) were collected and analyzed by SDS and native PAGE. Absorbance was monitored at 280 nm. Protein concentration was determined by Bradford assay (Pierce). Lethal factor (LF) was prepared as previously described [17].

2.2. SDS and native polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 10% Novex Tricine/SDS gels (Invitrogen, Carlsbad, CA). Proteins (2 μg each) were diluted with an equal volume of Novex Tricine SDS sample buffer and heated at 95 °C for 5 min. Electrophoresis was performed as recommended by the vendor. The gels were washed three times with deionized water for 5 min per wash, stained with Gel-Code Blue (Pierce Biotechnologies Inc., Rockford, IL) for 2 h, and destained with three changes of deionized water until the background staining was minimal. Western blot analysis was performed as previously described [16].

For native-PAGE, rPA samples (2 μg each) were diluted with an equal volume of Novex Tris–Glycine native sample buffer (Invitrogen) and loaded onto native PhastGels as recommended by the vendor (Amersham Biosciences). After electrophoresis, the PhastGels were stained with Gel-Code Blue as directed for larger gels (Pierce Biotechnologies Inc.). Gel images were captured on an HP Scanjet 7400C document scanner (HP, San Jose, CA) and stored as *.jpg files.

2.3. Protein isoaspartyl methyltransferase assay

The relative molar amount of isoaspartate present in rPA protein was measured by protein isoaspartyl methyltransferase assay (PIMT) using the ISOQUANT™ isoaspartate detection kit (Promega, Madison, WI), as performed in reversed phase-HPLC detection mode using a Sephasil Peptide C18 12-μm ST 4.6/250 column (Amersham Biosciences) heated to 37 °C on an Agilent 1100 liquid chromatography system and diode array detector (Agilent Technologies Inc., Wilmington, DE), essentially as described elsewhere [23].

2.4. Protein sequencing

N-terminal sequencing of purified proteins was performed using an Applied Biosystems Procise Model HT Sequencer (Applied Biosystems) using the manufacturer's standard protocol.

2.5. Vaccination and challenge of test animals

Pasteurella-free New Zealand White (NZW) rabbits, 2–3.5 kg, were obtained from Charles River Laboratories (Wilmington, MA). Three vaccine groups contained 30 ani-

mals each employing equal numbers of males and females per group. Four animals given phosphate-buffered saline (PBS) plus adjuvant were designated as controls. All animals were monitored for survival twice daily for 21 days after challenge. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

GMP grade rPA or each purified isoform was diluted in PBS and adsorbed in a 3% suspension of Alhydrogel (Superfos Biosector, Denmark), mixed thoroughly, and allowed to adsorb overnight at 4 °C. Rabbits were vaccinated i.m. in groups of 30 on days 0 and 28 with 10 μg of GMP grade rPA, isoform 1 or isoform 2 adsorbed to Alhydrogel. Each of four control animals (two males and two females) received 0.5 ml of PBS plus Alhydrogel. Rabbits were bled pre-vaccination, and at 2, 4, 6 and 8 weeks after they received the first vaccine dose. Serological assays were performed as described below.

Spores of the *B. anthracis* Ames strain were grown in Leighton and Doi medium, purified by centrifugation through 58% Renografin-76 (Superfos, Denmark) suspended in sterile water-for-injection containing 1% phenol and stored at 2–8 °C until used. Immediately before challenge, the spores were diluted in sterile water-for-injection to 3.12×10^8 colony-forming units (CFU)/ml, and heat-shocked at 60 °C for 45 min. This preparation was further diluted to 7.8×10^5 spores/ml and 0.2 ml were injected s.c. Aliquots of the spore dilutions (0.1 ml) were plated on 5% sheep blood agar plates to determine the actual challenge dose (CFU). We calculated that approximately129 LD_{50} had been administered.

2.6. Assay of rabbit anti-PA IgG by ELISA

Serum antibody titers to PA from vaccinated rabbits were determined using a quantitative anti-rPA IgG ELISA as described [24]. Briefly, microtiter plates (Immulon 2HB; Thermo-Lab Systems, Franklin, MA) were coated with rPA at 1 μg/ml overnight at 6–10 °C followed by incubation with standards and test sera at 37 °C for 1 h, then horseradish peroxidase-labeled goat anti-rabbit IgG (H + L) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 1 h at 37 °C, and finally 2,2′-azino-bis(3-ethylbenzthiozoline-6-sulfonic acid) (ABTS; Kirkegaard and Perry Laboratories) for 20–30 min at 37 °C. Plates were washed with PBS containing 0.1% Tween 20 between adding reagents and PBS containing 0.1% Tween 20 and 5% non-fat dry milk was used as dilutent for antibodies. The absorbance values were obtained using a BioTek 312e microplate reader (BioTek Instruments, Winooski, VT) with a 405-nm filter. The concentration of IgG was calculated by interpolating the average absorbance value for triplicate wells against the absorbance values of a standard curve generated from seven dilutions of affinity-purified rabbit anti-rPA IgG (KC4 Software, Biotek Instruments). The first data point that was within the linear portion of the standard curve was interpolated from the reference curve. Results from two separate assays are expressed as μg of IgG per ml and standard error (S.E.).

2.7. Anthrax cytotoxicity—neutralizing assay

The ability of antibodies to neutralize the cytotoxicity of lethal toxin for J774A.1 cells was measured using a cytotoxicity neutralization (CN) assay [24,25]. Briefly, standards and test sera were preincubated in 96-well microtiter plates (Corning Costar, Acton, MA) with purified rPA (50 ng/ml final concentration) and lethal factor (40 ng/ml final concentration) for 1 h at 37 °C. before transferring to a plate containing a monolayer of J774A.1 cells, plated the day before the assay with 5×10^4 cells per well. Plates were incubated for 4 h at 37 °C in 5% CO_2, followed by addition of 25 μl of 3-[4,5-dimethyl-thiazol-2-y-]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Company) at 5 mg/ml in PBS for 2 h before lysing the cells by adding 20% (w/v) SDS in 50% dimethylformamide, pH 4.7. Optical density readings were obtained at 570 nm with a reference wavelength at 690 nm on a BioTek 312e microplate reader (BioTek Instruments). The ratio between toxin plus antibody versus medium alone, expressed as a percentage of cell viability, was calculated for each dilution and plotted. The lethal toxin-neutralizing antibody titers of individual serum, calculated by four-parameter logistic regression analysis (XLFit Software, IDBS, Inc., Emeryville, CA), were expressed as the reciprocal of the antibody dilution preventing 50% of cell death (ED_{50}).

2.8. In vitro determination of biological activity of PA

Dilutions of rPA and selected fractions collected from the chromatographic separation of rPA isoforms were combined in triplicate with 40 ng/ml of LF (final concentration). Dilutions were prepared in Dulbecco's Minimal Essential Medium high glucose (D-MEM) containing 5% heat-inactivated fetal bovine serum, 4 mM glutamine and 100 U of Penicillin G and 100 μg of streptomycin per ml (D-MEM complete) supplemented with 25 mM HEPES. One-hundred microliters per well was then transferred to cell-culture treated, 96-well plates (Costar; Corning, NY) seeded with 5×10^4 of J774A.1 cells in 200-μl volumes 18–22 h before testing after aspirating off the medium. Cells were maintained in D-MEM complete. After incubating the plates for 4 h in a humidified incubator set at 37 °C and 5% CO_2, 25 μl per well of MTT at 5 mg/ml of PBS was added and the plates re-incubated for 2 h. Cells were then lysed and the insoluble reduced formazan was made soluble by adding 100 μl per well of lysing-solubilization buffer [26]. After an overnight incubation, the absorbance values at 570–690 nm for each well were measured by using a BioTek 312e

microplate reader (BioTek Instruments). The protein concentration of each PA test sample that resulted in 50% killing of cells in the presence of LF was calculated (EC_{50}) from four-parameter logistic regression analysis (XLFit software).

2.9. Statistical analysis

Log_{10} transformations were applied to all ELISA and CN assay titers. After transformation, the dependent variable met assumptions of normality and homogeneity of variance. These transformed ELISA and CN assay variables were used for all analyses. Mixed model analysis of variance (ANOVA) and Tukey post hoc tests were used to compare titers between groups. Pearson correlation was used to compare ELISA and CN assay titers. Survival rates were compared between groups and between genders within groups by Fisher Exact tests. All analyses were conducted using SAS Version 8.2 (SAS Institute Inc., SAS Online Doc, Version 8.2).

3. Results

3.1. Analysis and optimization of the purification process

Purified rPA contains differentially charged forms (isoforms) with equivalent relative mass [16]. Western blot analysis of samples collected at hourly intervals from an rPA production run revealed that two major isoforms are constitutively produced during fermentation even at the earliest time monitored (Fig. 1a). Purifying rPA by a method devised during research and development [16] resulted in final preparations that contained four predominant isoforms and several minor isoforms (Figs. 1b and 2a). These isoforms differed in net negative charge as revealed by fractionation with anion exchange chromatography (Fig. 1b). This step was incorporated into the GMP purification process using MacroPrepQ (BioRad), and further modified by adding an ammonium sulfate precipitation step instead of the 4 °C holding step described in the R&D method [16]. Adding butyl-hydrophobic interaction and anion-exchange chromatography steps (Fig. 2b) resulted in a GMP product containing only two major isoforms (Fig. 2a and c). The two predominant isoform bands in the GMP material appeared to have mobility equivalent to the two uppermost bands of the R&D material (Fig. 2a), suggesting they represented the same two isoform classes in both preparations.

The starting GMP grade rPA material used for isoform purification consisted of two isoforms as revealed by native PAGE (Fig. 2c, lane 1). Purifying the major upper isoform (isoform 1) and a minor lower isoform (isoform 2) was achieved by anion exchange chromatography (Fig. 2c, lanes 2 and 4); however, the process resulted in only 40% recovery of rPA. The remaining rPA consisted of a mixture of the two isoforms (Fig. 2c, lane 3). Attempts to separate the rPA isoforms by preparative electrophoresis resulted in less than

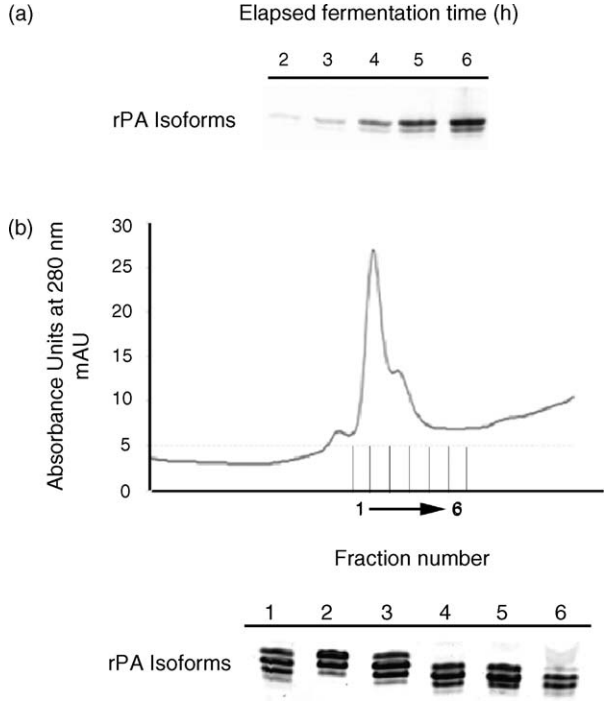

Fig. 1. (a) Analysis of culture supernatants from an rPA production fermentor run. Shown is a Western blot with anti-PA monoclonal antibody detection of rPA isoforms being constitutively produced during the course of fermentation. (b) Chromatogram of R&D grade rPA purified by anion exchange and corresponding native PAGE of fractions eluted by a gradient of increasing salt concentration. Fraction 1 consists of a mixture of four isoforms, whereas fraction 6 contains mostly isoforms 3 and 4 which are less biologically active than fraction 1 (EFT: elapsed fermentation time).

10% protein recovery due to protein precipitation (results not shown). Protein sequencing revealed that isoforms purified by anion exchange chromatography had N-termini (NH2-EVKQENRLLN-COOH) identical to that of the predicted protein sequence, and to that of the GMP grade material. These results suggested that the micro-heterogeneity observed by native PAGE was not due to N-terminal degradation of rPA. Sufficient protein in each of these fractions was recovered for biological studies.

3.2. Analysis for isoaspartate

The relative molar amount of isoaspartate (isoAsp) was measured and compared between the research and GMP grade protein preparations as an appraisal for possible amino acid deamidation. Analysis by PIMT assay revealed that both rPA preparations contained isoAsp, with the R&D grade material containing four times more measured picomols of isoAsp per picomol of protein (Table 1). Assuming an average conversion ratio of 0.775 mol isoAsp per mole of convertible amino acid (glutamine and asparagine), and assuming asparaginyl residues provided the entire source of detected isoAsp, these measurements agree with an extrapolated 14 deamidated asparagines in the GMP grade material (Table 1 and footnotes). While deamidation was not directly demon-

Table 1
Measurement of isoaspartate in rPA preparations by PIMT assay

Sample	Number of major isoforms	Peak area per pmol protein	Measured pmol isoAsp per pmol protein[a]	Estimated pmol deamidation per pmol protein[b]	Estimated number of deamidated asparaginyl residues per rPA protein[c]
IsoAsp-DSIP[d]	N/A	0.7[a]	1	1	N/A
R&D grade rPA	>4	0.473	0.68	0.877	59
GMP grade rPA	2	0.114	0.163	0.210	14

[a] Peak area per picomole of IsoAsp-DSIP standard is used for normalization, as instructed by the kit vendor (Promega).
[b] One mole of unstable succinimide intermediate, resulting from 1 mol of deamidated asparaginyl and glutaminyl residues, spontaneously resolves into 0.7–0.85 mol (mean 0.775) of isoaspartyl residue. From this an averaged conversion factor of 1.29 is applied to estimate the quantity of deamidated residues per measured isoaspartate.
[c] Calculated as the product of estimated deamidation and the total number (67) of asparaginyl residues in rPA, assuming that deamidation of rPA derives only from asparaginyl sources as glutaminyl deamidation is typically 300-fold less frequent than that for asparaginyl, and that these asparaginyl sites are each completely deamidated.
[d] IsoAsp-DSIP is the standard positive control peptide provided by the kit vendor and contains one isoaspartyl residue.

strated, these data provided indirect evidence for the presence of deamidated asparaginyl and/or glutaminyl residues in purified rPA, and showed that isoform complexity correlates with the amount of isoAsp, which is likely to be deamidation. Elucidation of the precise amino acids affected in rPA will be described elsewhere (Powell et al., in preparation).

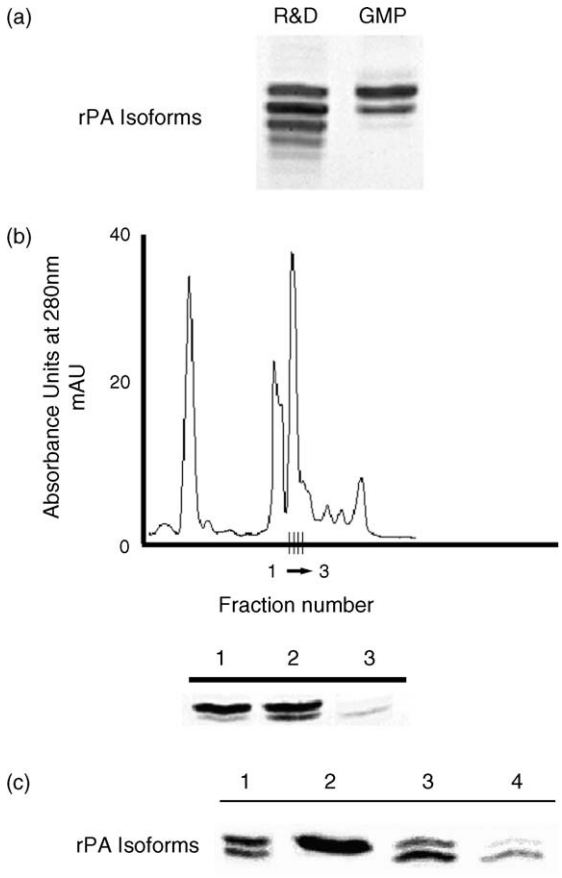

Fig. 2. (a) Analysis of R&D grade rPA compared to GMP grade rPA by native PAGE and Gel-Code Blue staining. The R&D grade rPA contains four or more isoforms, while the GMP grade rPA contained only two. (b) Chromatogram of GMP grade rPA purification by anion exchange chromatography and corresponding native PAGE of fractions 1–3. (c) Native PAGE analysis of GMP rPA used in this study. Starting material (lane 1), isoform 1 (lane 2) and isoform 2 (lane 4) enriched by anion exchange chromatography for animal efficacy studies. A mixture of isoforms 1 and 2 which did not separate by this technique is shown in lane 3.

3.3. Immunogenicity of rPA isoforms

Rabbits inoculated with isoform 1 or isoform 2 showed no significant difference in anti-PA IgG antibody titers between the two isoforms of rPA ($p = 0.8436$, Table 2). Anti-PA IgG antibody titers from isoform 1 showed a significantly higher anti-PA IgG response when compared to GMP rPA ($p = 0.0411$) responses. Anti-PA IgG antibody titers from isoform 2 also showed a significantly higher anti-PA IgG response when compared to GMP rPA ($p = 0.0092$) responses. The difference in quantitative ELISA titer may be explained by the inherent variability in animal responses. Comparing ED_{50} CN titers between groups (Table 2) showed that there were no significant differences between groups ($p = 0.2357$).

3.4. In vitro biological activity of rPA isoforms

There was no significant difference between the biological activities of rPA isoforms 1 and 2 purified from GMP grade material in the presence of LF as revealed by cytotoxicity assays ($p = 0.3943$) (Table 3). Previous studies of R&D grade rPA, however, revealed that chromatographic fractions consisting of mixtures of more negatively charged isoforms (lower isoforms 3–5 by native PAGE), were less biologically active than GMP grade rPA (data not shown).

Table 2
Immunogenicity and protective efficacy of individual rPA isoforms against *B. anthracis* infection in rabbits[a]

Vaccine	Anti-PA ELISA[b]	CN titer[c]	Survival
PBS/AlOH	0	50	0/4 (0%)
Isoform 1, 10 μg	322.87 (1.14)	6493 (1.10)	28/30 (93%)
Isoform 2, 10 μg	350.30 (1.09)	7587 (1.06)	29/30 (96%)
GMP rPA, 10 μg	224.95 (1.09)	6331 (1.09)	28/30 (93%)

[a] Rabbits were inoculated on days 0 and 28 with 10 μg of isoform 1, isoform 2 or GMP rPA, and challenged as described in Section 2.
[b] Geometric mean anti-PA IgG (μg/ml) ELISA and S.E. at 8 weeks.
[c] Geometric mean ED_{50} CN titer and S.E. at 8 weeks.

Table 3
Biological activity of GMP grade rPA and purified isoforms in the presence of *B. anthracis* lethal factor (LF) as measured by cytotoxicity

Sample	EC_{50} (ng/ml protein)
GMP rPA	16.72
Isoform 1	12.10
Isoform 2	10.38

3.5. Protection against lethal challenge by rPA isoform vaccines

New Zealand White rabbits were vaccinated i.m. in groups of 30 on days 0 and 28 with 10 μg of GMP grade rPA, isoform 1 or isoform 2 adsorbed to Alhydrogel. The negative control group received Alhydrogel plus PBS. Animals were challenged parenterally with a lethal dose (129 LD_{50}) of *B. anthracis* spores of the Ames strain 10 weeks after the first vaccine dose. All four animals in the control group that received PBS plus Alhydrogel succumbed within 4 days of challenge. The protective efficacies of both isoforms and GMP grade rPA were all greater than 90% (Table 2). Fisher Exact tests revealed no significant differences in survival rates among vaccinated groups ($p = 1.00$).

4. Discussion

The currently licensed anthrax vaccine, BioThrax (BioPort Corporation, Lansing, MI), consists primarily of PA. BioThrax is produced from the fermentor culture filtrate of a toxigenic, non-encapsulated strain of *B. anthracis*. BioThrax contains small, but varying quantities of other bacterial components. A second-generation vaccine currently undergoing clinical trials is a purified recombinant PA product obtained from cultures of an avirulent, asporogenic and non-toxigenic strain of *B. anthracis*. The fermentation, purification, and characterization of rPA produced from the recombinant strain were described by Farchaus et al. [16]. In preparation for clinical trials, a robust production process was developed which is amenable to the GMP guidance of the U.S. Food and Drug Administration (FDA). However, although GMP grade rPA appears as a M_r 83,000 monomer by SDS PAGE, it separates by native PAGE or analytical capillary isoelectric focusing into two discrete isoforms with isoelectric points of 5.1 and 5.2.

Deamidation of asparagine residues is one of the most common post-translational modifications and results in protein isoforms. Aspartate and asparagine residues are most reactive, leading to the formation of a succinimide. Succinimides are unstable intermediates that undergo spontaneous hydrolysis to a mixture of aspartyl and isoaspartyl residues. Exposure to alkaline pH results in an increased rate of succinimide formation due to greater deprotonation of the peptide bond nitrogen. When rPA was maintained at alkaline pH for 16 h or more at 4 °C, four or more isoforms could be detected by native PAGE [16]. Deamidated products can be detected by selective methylation of isoAsp sites with protein isoaspartyl methyltransferase and HPLC separation and UV measurement of a co-product of the reaction [23]. While strongly indicative of the presence of deamidation, assays for isoaspartate are not definitive for deamidation since isoaspartate can also derive from isomerization of aspartate. Moreover, the presence of isoaspartate cannot distinguish between glutamine versus asparagine sources of the product. In this study, we revealed that GMP grade rPA contained isoAsp, suggesting deamidation, and this level was significantly less than that observed in the R&D grade rPA. A follow-on study that defines the identity and extent of post-translational modifications for amino acids in rPA by LC/MS/MS will be described separately (Powell et al., in preparation). A reduction or complete loss of in vitro or in vivo biological activities has been reported for a variety of deamidated proteins [27,28], while others appear unaffected [29]. It is therefore important to establish methods for evaluating the effects of deamidation on the biological activities and antigenicities of vaccine candidate polypeptides. Previous studies with R&D grade protein demonstrated that rPA containing mixtures of the more negatively charged isoforms 3 and 4 were less biologically active than mixtures of rPA containing isoforms 1 and 2 (Little, personal communication, results not shown). In this study, the in vitro biological activities of purified isoforms 1 and 2, compared to GMP grade rPA were equivalent. A possible explanation for why rPA preparations rich in more negatively charged isoforms are less biologically active in vitro may be the improper folding of one or more of the four functional domains. Elucidation of the extent of deamidation within each of the rPA domains, i.e., receptor-binding domain, or lethal/edema factor binding domain [30–36], may provide additional information, but is beyond the scope of this report. GMP grade rPA does not contain the more negatively charged isoforms present in the R&D grade material, which resulted in observable differences in biological activity.

We conducted an rPA isoform vaccine efficacy study that required sizeable amounts of each of the two isolated isoforms. Separating individual isoforms proved to be extremely difficult by preparative isoelectric focusing (IEF) on immobilized pH gradients (due to protein precipitation at pH values near the pI of rPA), or by anion exchange chromatography. Both techniques resulted in substantial losses of rPA (90 and 60%, respectively). During anion exchange chromatography, the application of a linear gradient of increasing salt concentration resulted in the elution of isoform 1 (peak front), followed by the majority of the rPA as a mixture of isoforms 1 and 2 (middle), and then isoform 2 (trailing edge). Baseline resolution of the isoforms was not achieved by this method, and only by collecting small fractions across the peak were we able to isolate enough of each isoform for the animal protective efficacy studies.

Rabbits, used extensively in anthrax research, are sensitive to *B. anthracis* and are a good predictor of vaccine efficacy in rhesus monkeys [14]. We modeled our isoform protec-

tive efficacy study based on a titration study with rabbits previously conducted at USAMRIID. In that study, 10 New Zealand white rabbits per group received 50, 5 or 0.5 μg of rPA adsorbed to Alhydrogel. A lethal aerosol challenge with Ames strain spores (63 LD_{50}) resulted in a 100, 90 and 60% survival per group, respectively. The design of this study was based on those results.

There were no significant differences in biological activity or vaccine efficacy between the two isoforms. Thus, as expected, the biological activity and vaccine efficacy of the two isoforms did not differ from that of the GMP rPA.

The final phase in the new anthrax vaccine development plan is to conduct clinical trials. A two-part phase 1 escalating-dose study in humans of the safety and immunogenicity of the rPA vaccine is in progress. The main objective of this study was to determine if purified rPA isoforms differ in their ability to elicit protective immunity in rabbits challenged parenterally with *B. anthracis* Ames strain spores, and whether purifying individual isoforms is necessary for the new-generation vaccine. The results presented here strongly suggest that purifying individual rPA isoforms or controlling the relative amounts of the isoforms is unnecessary, as both had equivalent biological activity and protective vaccine efficacy in rabbits.

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